Characterization of Monoclonal Antibodies Generated to the Cornified Envelope of Human Cultured Keratinocytes

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The cornified envelope of keratinocytes is an insoluble structure formed beneath the plasma membrane at the base of the stratum corneum. It is made by cross-linking precursor proteins by a membrane-associated transglutaminase. We have prepared monoclonal antibodies to the cornified envelope of cultured human keratinocytes and used these to identify precursor proteins using Western blotting. We have uncovered a number of precursors including involucrin and a 195 kD membrane-associated protein, which had previously been reported. Antibodies to these precursors, with the exception of the one to involucrin, reacted with the epidermis of other mammalian species, suggesting structural conservation in at least some envelope components. *J Invest Dermatol 89:454–459, 1987*

The cornified envelope is a highly insoluble intracellular structure that is found in the epidermis of mammalian skin, the external root sheath and cuticle of hair, and nail (also hoof) [1–3]. It is also present in certain stratified epithelia. Its insolubility in denaturing solvents results from the presence of ϵ-(γ-glutamyl)lysine cross-links that are formed by a membrane-associated transglutaminase [4,5].

A soluble transglutaminase substrate, involucrin, has been isolated from cultured human keratinocytes and it has been postulated that this is the major precursor of human cornified envelope both in vivo and in vitro [6]. Additional precursors of the membrane have also been described [7–10], and a group of investigators [10] has suggested that one of these, termed keratolinin, is the major in vivo precursor of the human epidermal cornified envelope.

The initial studies on the precursors of the envelope used labeled transglutaminase substrates [9] or the disappearance of proteins [7] and the formation of high–molecular weight aggregates [11]. More recently investigators have developed polyclonal or monoclonal antibodies against whole cells or cell fragments to identify putative precursors of the envelope [8,12].

We report the use of cornified envelopes derived from human cultured keratinocytes to generate monoclonal antibodies for detection of envelope precursors. A large number of antibodies were found by indirect immunofluorescence, and many of these showed the peripheral-type stain observed with involucrin. The antigens responsible for some of these reactions could be identified and these are described.

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Abbreviations:
DTT: dithiothreitol
EDTA: ethylenediamine tetracetate
FITC: phenylisothiocyanate
IIF: indirect immunofluorescence
PAGE: polyacrylamide gel electrophoresis
PMSF: phenylmethylsulfonyl fluoride
SDS: sodium dodecyl sulfate

MATERIALS AND METHODS

Tissue Human foreskin keratinocytes were cultured using the method of Rheinwald and Green [13], with slight modification [14]. Stratified cultures 1–2 weeks beyond confluence were rinsed extensively with serum-free media and then harvested with a plastic policeman.

Isolation of Cornified Envelope Human cultured keratinocytes were homogenized at a ratio of 20:1 (v/w) in 100 mM Tris-HCl, pH 9.0, containing 2% sodium dodecyl sulfate (SDS) and 10 mM dithiothreitol (DTT), and the homogenate was heated with stirring for 2 h at 55°C. The suspension was clarified by centrifugation at 50,000 *g* for 30 min and the pellet reextracted as above 5 additional times. Protein was undetectable in the final supernatant by the Lowry method [15]. The insoluble residue was freed from SDS by washing with 95% ethanol, and then resuspended in 100 mM sodium bicarbonate buffer. To quantitate the amount of cornified envelope protein, an aliquot was hydrolyzed for 24 h in 6 N HCl, and the free amino acids were measured by the ninhydrin method using L-leucine as a standard [16]. The microgram of amino acid was assumed to equal the microgram of cornified envelope material.

A suspension containing 50 μg of purified cornified envelope in 0.2 ml of Freund's incomplete adjuvant was injected i.p. into 6 BALB/c mice. The injections were repeated every other week until the sera showed a reaction to human epidermis by indirect immunofluorescence (IIF). The mice were rested for 2 months, during which time two animals died. The remaining 4 were immunized on three successive days, and on the fourth day they were sacrificed, their spleens removed and pooled, and the cells from the spleens fused with NS-1 mouse myeloma cells in the presence of 37% polyethylene glycol (MW 1000). Monoclonal antibodies were developed as described previously [17,18]. Hybridoma cultures were screened for antibodies that gave a reaction with human epidermis by IIF.

V8 Protease Digestion of Cell Envelopes Human cell envelopes were suspended at 0.5 mg/ml in 125 mM Tris, pH 6.8,

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Table I. Types of Staining Patterns Observed by IIF and Number of Wells Containing Hybridoma Cells Giving a Particular Pattern

Pattern	Number of Reactive Wells
Cytoplasmic	
whole epidermis	14
basal layer	9
suprabasal	5
stratum corneum	24
Peripheral	42
Basement membrane	2
Nuclear	9
Total	105

Table II. Polypeptides Identified by Immunoblotting Using Supernatants of Cloned Cells that Gave a Peripheral Stain With Human Epidermis by IIF

Antigen Identified	Number of Clones[a]
Involucrin	5
195 kD band	4
~82 kD bands	6
Basic polypeptides (23)	1
Keratins[b]	12
Other[c]	3
Total	31

[a]Number of clones giving a particular pattern.
[b]Weak reactions to keratin bands strongly stained by Fast Green, which may be nonspecific.
[c]One was to a band of slightly greater molecular weight than involucrin and one to a band of slightly smaller molecular weight than involucrin. The third was in the 30 kD range. None of these have been further characterized because the intensity of the reaction was not strong.
IIF, indirect immunofluorescence.

with 0.5% SDS and 10% glycerol [19]. V8 protease was added to the suspensions to a final concentration of 100 μg/ml and then incubated at 37°C for varying times. After incubation the samples were made 2% in SDS and 10% in β-mercaptoethanol and boiled for 2 min. After centrifugation at 10,000 *g* for 15 min, the supernatants were prepared for electrophoresis.

Additional Procedures Cells were extracted by homogenization at 0°C at a ratio of 10:1 (v/w) in 50 mM Tris, pH 7.6, containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) (Tris-EDTA) and centrifugation at 50,000 *g* for 30 min. The pellets were extracted in 0.1 M Tris, pH 9.0, with 6 M urea and 0.1 M β-mercaptoethanol (TUM) for 4 h at 4°C, and then centrifuged at 50,000 *g* for 30 min. Alternatively, the cells were extracted in 100 mM Tris, pH 9.0, containing 1% SDS, 10 mM DTT and 0.1 mM PMSF (Tris-SDS-DTT) at 50°C. Electrophoresis was done in 7% or 15% polyacrylamide slab gels,

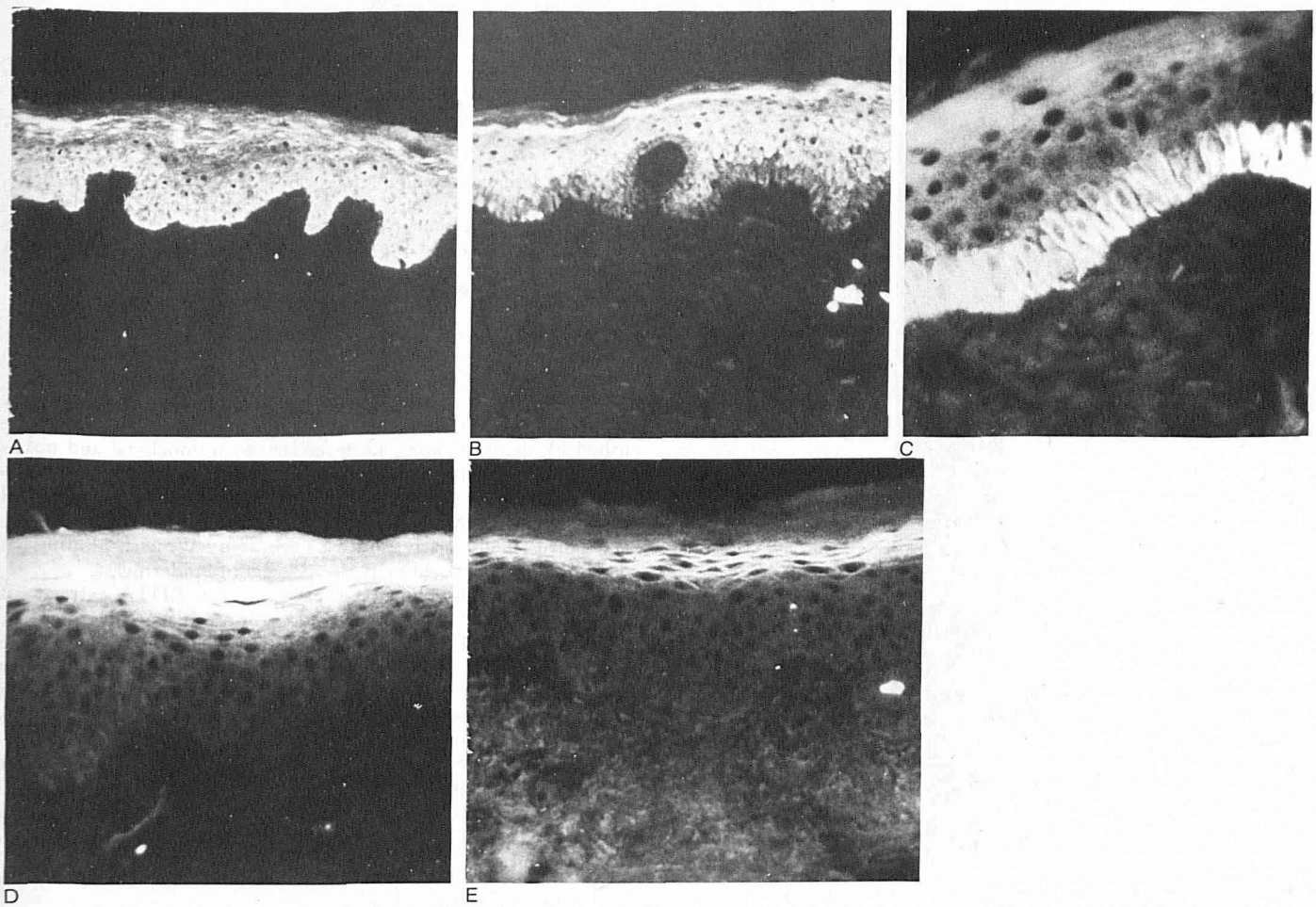

Figure 1. Examples of various indirect immunofluorescence staining patterns of human epidermis using some of the monoclonal antibodies listed in Table I. *A*, Cytoplasmic staining of whole epidermis. *B*, Suprabasal cytoplasmic staining. *C*, Basal layer cytoplasmic staining. *D*, Stratum corneum staining. *E*, Peripheral staining of upper epidermis.

according to Laemmli [20]. Transfer of separated polypeptides from polyacrylamide gels to nitrocellulose was accomplished by the method of Towbin and colleagues [21], using the Bio-Rad Trans Blot apparatus. Indirect immunofluorescence was done as previously described [22]. Secondary antibodies for IIF were FITC conjugated goat IgG to mouse IgG diluted 1 to 30. In the case of immunoblotting, both FITC-conjugated and HRP-conjugated goat antibodies were used at dilutions of 1:60 and 1:2000, respectively. A mouse polyclonal antibody to a 195 kD submembranous protein precursor of the cell envelope was kindly supplied by Dr. Marcia Simon [7].

RESULTS

Four thousand wells were seeded with cells from the fusion and 1025 were found to have sufficient growth to warrant screening. The types and number of IIF patterns observed are shown in Table I. The stratum corneum reactions were described as cytoplasmic because the entire layer was fluorescent in most cases. Because of the compactness of the cells, however, peripheral staining might have been missed. Examples of some of the staining reactions are shown in Fig 1.

All the hybridoma lines that gave a peripheral stain were recloned and tested by Western blotting to Tris-EDTA or Tris-SDS-DTT extracts of cultured human keratinocytes. There were four unique intense blotting patterns observed (Figs 2–5), which are discussed below in detail, but not all the antibodies showed one of these. A number of antibodies gave a weak reaction to one of the strong keratin bands. The number of the different types of blotting reactions are shown in Table II.

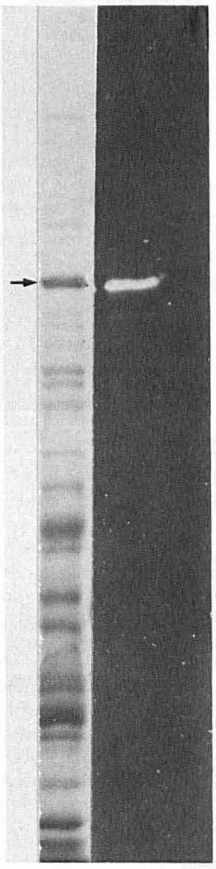

Figure 2. Immunoblot of a Tris-EDTA extract of human cultured keratinocytes separated by 7% SDS PAGE. The *left lane* was stained with Fast Green and the immunoreactive band (*arrow*) has a molecular weight of 143 kD. The *right lane* is the immunoblot using a FITC conjugated second antibody.

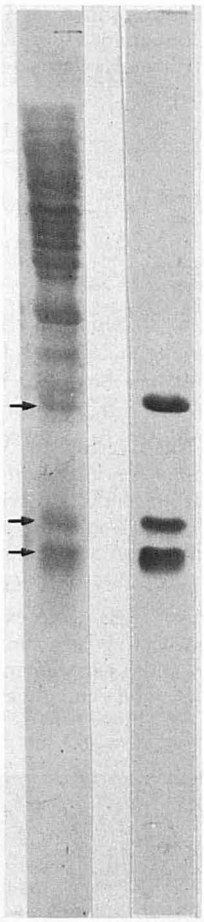

Figure 3. Immunoblot of a Tris-EDTA extract of human cultured keratinocytes separated by 15% SDS PAGE. The *left lane* was stained with Fast Green and the immunoreactive bands (*arrows*) have molecular weights of 14.9 kD, 16.8 kD, and 24.8 kD. The *right lane* is the immunoblot using an HRP conjugated second antibody. The smearing of the 14.9 kD band is seen in some preparations and may be due to degradation.

One of the reactions (Fig 2) was to involucrin, which had a molecular mass of 143 kD. The identity of the band was established by demonstrating its reactivity to monoclonal and polyclonal antibodies previously prepared to involucrin [12]. The previously prepared monoclonal and two of the new monoclonal antibodies were tested against bovine, rat, and mouse epidermis, and failed to show a response by IIF or by Western blotting of Tris-EDTA or Tris-SDS-DTT extracts of epidermis.

Another reaction (Fig 3) observed in Tris-EDTA extracts of cultured cells was to 3 polypeptides of molecular masses of 14.9, 16.8, and 24.8 kD, which have previously been shown to have a pI close to 9.0 by two dimensional electrophoresis [23]. The antibody showed peripheral staining of bovine and rat epidermis by IIF, and Tris-EDTA extracts of bovine and rat epidermis showed reactive bands of 22 kD and 29 kD, respectively.

A third reaction (Fig 4) was found in Tris-EDTA extracts of cultured cells, but was much more intense in a Tris-SDS-DTT extract. The reaction showed 2 or 3 closely positioned bands with molecular masses of about 82 kD. This antibody reacted peripherally with bovine and rat epidermis by IIF, and Tris-SDS-DTT extracts of bovine and rat epidermis showed reactive bands of 97 kD and 78 kD, respectively.

The fourth reaction (Fig 5) observed was to a 195 kD band and like the third was detected in the Tris-EDTA extract of cultured cells, but was more intense in the Tris-SDS-DTT extract. A

previously described antibody to a 195 kD submembranous protein [7] gave a reaction at the same position (Fig 5). Our antibody also reacted to bovine epidermis by IIF and to a component of about 200 kD in a Tris-SDS-DTT extract of bovine epidermis by Western blotting.

Some hybridoma culture supernatants gave a weak reaction to the heaviest keratin bands. Very likely these were nonspecific, since they were weak in intensity and could be observed with a number of other indifferent monoclonal antibodies.

The three antibodies that gave a weak reaction and are listed as "others" were not further studied.

Following V8 protease digestion of cornified envelopes for 30, 60, and 120 min, the solubilized proteins were examined by Western blotting using the antibodies described in Figs 2–5. A reaction was observed at 60 and 120 min at the top of the separating gel, using the antibody against the basic polypeptides. When the experiment was repeated doing a 72-h digestion, a very strong reaction was observed with the antibody to the basic polypeptides and a weak but definite reaction with the antibody to involucrin (Fig 6). No reactions were observed to the other antibodies.

DISCUSSION

These studies have shown the value of using purified cornified envelopes to elicit antibodies to identify putative precursors of the cornified envelope. The major problem we encountered was the cumbersome assay that was needed to identify the antibodies. This, however, was the only technique that gave the required sensitivity and specificity. We have found that absorbent assays gave too many false negative and false positive reactions and, in the end, were not less time consuming.

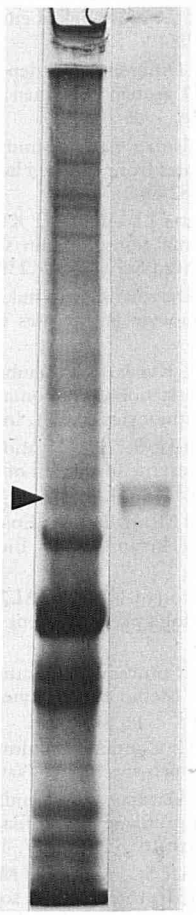

Figure 4. Immunoblot of a Tris-SDS-DTT extract of human cultured keratinocytes separated by 7% SDS PAGE. The *left lane* was stained with Fast Green and the immunoreactive bands (*arrowhead*) have a molecular weight of about 82 kD. The *right lane* is the immunoblot using HRP conjugated second antibody. The top of the stacking gel is indicated by a drawn line.

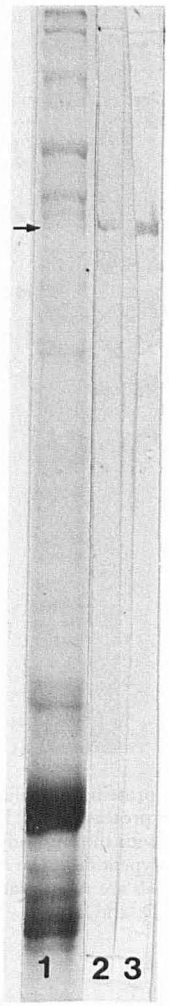

Figure 5. Immunoblot of a Tris-SDS-DTT extract of human cultured keratinocytes separated by 7% SDS PAGE. *Lane 1* was stained with Coomassie brilliant blue, and the immunoreactive band (*arrow*) has a molecular weight of 195 k. *Lane 2* was reacted with the monoclonal antibody reported in this study, and *lane 3* with the antibody provided by Dr. Marcia Simon. Both antibodies stain the same band.

Two of the precursors observed with greatest frequency, involucrin and the 195 kD band, had been previously discovered as transglutaminase substrates that became cross-linked or incorporated labeled amines [7,9]. The third, the 82 kD, was a previously unrecognized group of polypeptides that, up to now, had escaped detection. That these were present in human epidermis was confirmed by the IIF studies as well as immunoblot studies of Tris-EDTA-DTT extracts of epidermis (unpublished observations). It remains to be established if the multiple bands are distinct polypeptides, degradation products, or posttranslationally modified proteins. Additionally, it must be determined whether they are only ϵ-lysine donors, which might explain why they were not previously recognized.

Only one of these three monoclonal antibodies reacting to the precursors was specific for human keratinocytes, the antibody to involucrin. The antibody to the 82 kD polypeptides reacted by IIF with bovine and rat epidermis and the 195 kD one reacted with at least bovine epidermis. These results suggest that proteins

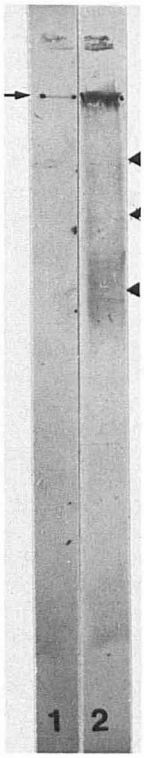

Figure 6. Immunoblots of proteins solubilized by treatment of purified cornified envelopes with V8 protease for 72 h and separated by 15% SDS PAGE. *Lane 1* was reacted with the antibody to involucrin and *lane 2* to the antibody to the basic polypeptides. The *arrow* indicates the top of the separating gel. The *arrowheads* are molecular weight markers and are in decreasing size 66 kD, 45 kD, and 29 kD.

with antigenic determinants similar to these two precursors may be widely distributed in mammalian species. It has been postulated that involucrin, on the other hand, is present only in higher primates [24]. A recent report, however, showed that extracts of bovine epidermis reacted weakly with a polyclonal antibody to involucrin and a polyclonal antibody to the cornified envelope of bovine keratinocytes reacted with human involucrin [25]. Similar cross-reactions have not been observed with rat and mouse epidermis. It is not clear at the present time how similar the bovine protein is to human involucrin or whether the immunologic reactivity is merely the result of a common structural configuration that is recognized by the antibodies.

The antibody to the basic polypeptide was found in only one clone; however, this may have been a result of a technical difficulty with the assay. We have recently observed (unpublished observations) that the antigen is not as stable in tissue sections as we had initially believed, and in the process of assaying so many wells, we may have missed positive clones. Unfortunately, the cells from wells where supernatants were found not to react to human epidermis by IIF were discarded, so that retesting is not possible.

The number of clones giving rise to a particular antibody can not necessarily be used as evidence for the relative importance of the protein eliciting the response as an envelope component. The various components may differ in their ability to elicit an immune response. In addition, location within the envelope and how the protein is cross-linked may obviously have a profound effect on the elicitation of an immunologic response.

Finally, there is no obvious explanation for the finding of a large number of antibodies that reacted by IIF to human epidermis, but for which a specific solubilized precursor could not be identified. Although there were some antibodies that gave a weak reaction to keratin, we tentatively regard these as nonspecific, since indifferent antibodies may give such a reaction. These antibodies may be to structural configurations generated in the process of cross-linking or posttranslational modifications that we have not yet identified. These antibodies may prove useful, therefore, in studies of envelope assembly using purified precursors and transglutaminase.

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